PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 4394PTWO - DZ	**FOR FURTHER ACTION**	See Form PCT/IPEA/416
International application No. PCT/EP2004/000789	International filing date *(day/month/year)* 29.01.2004	Priority date *(day/month/year)* 29.01.2004
International Patent Classification (IPC) or national classification and IPC INV. C12N15/87		
Applicant BIOSILAB S.R.L et al.		

1. This report is the international preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 5 sheets, including this cover sheet.
3. This report is also accompanied by ANNEXES, comprising:
   a. ☒ *sent to the applicant and to the International Bureau)* a total of 3 sheets, as follows:
      ☒ sheets of the description, claims and/or drawings which have been amended and are the basis of this report and/or sheets containing rectifications authorized by this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions).
      ☐ sheets which supersede earlier sheets, but which this Authority considers contain an amendment that goes beyond the disclosure in the international application as filed, as indicated in item 4 of Box No. I and the Supplemental Box.
   b. ☐ *(sent to the International Bureau only)* a total of (indicate type and number of electronic carrier(s)) , containing a sequence listing and/or tables related thereto, in electronic form only, as indicated in the Supplemental Box Relating to Sequence Listing (see Section 802 of the Administrative Instructions).

4. This report contains indications relating to the following items:

☒	Box No. I	Basis of the report
☐	Box No. II	Priority
☐	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☐	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☐	Box No. VI	Certain documents cited
☐	Box No. VII	Certain defects in the international application
☐	Box No. VIII	Certain observations on the international application

Date of submission of the demand	Date of completion of this report
07.11.2005	21.04.2006
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Friedrich, C Telephone No. +49 89 2399-7721

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/EP2004/000789

Box No. I Basis of the report

1. With regard to the **language**, this report is based on

   ☒ the international application in the language in which it was filed

   ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of:
   - ☐ international search (under Rules 12.3(a) and 23.1(b))
   - ☐ publication of the international application (under Rule 12.4(a))
   - ☐ international preliminary examination (under Rules 55.2(a) and/or 55.3(a))

2. With regard to the **elements*** of the international application, this report is based on *(replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report)*:

Description, Pages	
1-13	as originally filed
Claims, Numbers	
1-25	received on 07.11.2005 with letter of 02.11.2005
Claims, Pages	
14-16	as originally filed
Drawings, Sheets	
1/8-8/8	as originally filed
Drawings, Figures	
1-8	as originally filed

   ☐ a sequence listing and/or any related table(s) - see Supplemental Box Relating to Sequence Listing

3. ☐ The amendments have resulted in the cancellation of:
   - ☐ the description, pages
   - ☐ the claims, Nos.
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

4. ☐ This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).
   - ☐ the description, pages
   - ☐ the claims, Nos.
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

* *If item 4 applies, some or all of these sheets may be marked "superseded."*

Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-23
	No:	Claims	24-25
Inventive step (IS)	Yes:	Claims	1-23
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-25
	No:	Claims	

2. Citations and explanations (Rule 70.7):

see separate sheet

Reference is made to the following documents:

D1: Huang and Rubinsky, 2001. Microfabricated electroporation chip for single cell membrane permeabilization. Sensors and Actuators A, 89:242.

D2: Lin et al. 2003. A microchip for electroporation of primary endothelial cells. Sensors and Actuators A, 108:12.

Introduction

The present application concerns a biochip for electroporation of single cells. The gist of the application appears to be the possibility to transfer signals to pre-selected single microelectrodes.

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Novelty, Art.33(1) and (2), PCT

1.1. The prior art does not disclose biochips for electroporation with a control system that permits to transfer signals to pre-selected single microelectrodes. Subject-matter of claims 1-23 therefore appears to be new (Article 33(2) PCT).

1.2. Electroporated cells as referred to in claims 24 and 25 are not new over the prior art, as the method of electroporation does not confer any technical feature specific to said method. The cells are e.g. identical to elctroporated cells disclosed in D1 or D2.

2. Inventive Step, Art.33(1) and (3), PCT

The problem to be solved by the present invention may be regarded as the electroporation of pre-selected cells. The solution to this problem proposed in claims 1-23 of the present application is considered as involving an inventive step (Article 33(3) PCT) for the following reasons:

The closest prior art (D1) discloses the electroporation of single cells in a flow through system. In D2 microchips with interdigitating electrodes are disclosed. However, nowhere in the prior art is disclosed the possibility to transfer signals to pre-selected single microelectrodes. The solution put forward in the present application thus allows to electroporate selected single cells instead of just single cells. This selection is neither

disclosed nor suggested in D1 or in D2.

3. Industrial Applicability, Art.33 (1) and (4), PCT

Subject-matter of the present application appears to be industrially applicable under Art.33(1) and (4), PCT.

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NEW SET OF CLAIMS

(55)

1. Apparatus for electroporation comprising a wave generator, a biochip containing an array of microelectrodes and a control system that permits to transfer the signal to a pre-selected single microelectrode of the biochip.
2. Apparatus according to claim 1 characterised in that the control system consists of a personal computer equipped with a software program capable of designing various waveform signals and a switching system controlling the wave generator output.
3. Apparatus according to claims 1 and 2 characterised in that the biochip comprises an array of microelectrodes of a size comparable to the cell to be electroporated and each of said microelectrode being driven separately from the others allowing very precise and punctual control of the electroporation process.
4. Biochip comprising an array of individually driven microelectrodes (20) comprised on a suitable insulating layer mounted on a solid substrate; means to electrically connect said microelectrodes to a switching system; a cell culture chamber where the cells can be grown and adhere in contact with said array of microelectrodes on a surface formed by said insulating layer containing said array of microelectrodes on said solid substrate.
5. Biochip according to claim 4 comprising a semiconductor substrate as solid substrate covered with an insulating layer (27) comprising said array of individually driven microelectrodes (20) of a size comparable to the cell to be electroporated, and mounting a cell culture chamber (24) with an opening (26) mounted, in turn, on a support (21) made of dielectric material, said microelectrodes (20) being electrically connected via conductive traces (28) to conductive pads (29) electrically connected, in turn, to a couple of external parallel connectors (22) through wire bonding (23) covered by the outer portion of the cell culture chamber (24) encircling the opening (26), being said cell culture chamber (24) with the opening (26) mounted over the top of the said semiconductor substrate covered with an insulating layer (27), both attached on the dielectric support (21).

6. Biochip according to claim 5 comprising two further electrodes (25) integrated in the semiconductor substrate covered with an insulating layer (27), and acting as ground reference.
7. Biochip according to claim 5 wherein the semiconductor substrate covered with an insulating layer (27) is a silicon substrate covered with a insulating layer preferentially of SiO_2.
8. Biochip according to claim 5 wherein these solid substrates are transparent.
9. Biochip according to claim 5 wherein the dielectric support is vetronite, glass or ceramic.
10. Biochip according to claim 5 wherein the microelectrodes of the array (20) have a size with a surface of at least ten per cent of the total cell membrane and preferably a diameter ranging from 1 µm to 50 µm.
11. Biochip according to claims 4 - 10 wherein the microelectrodes are of conductive or capacitive type.
12. Microelectrodes according to claim 11 consisting of conductive microelectrodes obtained over a silicon substrate (31) covered with a insulating layer preferentially of SiO_2 (32), said microelectrodes and their connecting traces (38) being made by a "sandwich" of two titanium nitride (TiN) layers (33) and an aluminium layer (34), covered with a gold layer (37) on their active surface.
13. Microelectrodes according to claim 11 wherein said microelectrodes are realised using Metal Oxide Semiconductor (MOS) technology.
14. Microelectrodes according to claim 13 consisting of a silicon p-type substrate (40) in which two n-doped regions, drain (41) and source (42), are implanted with conventional microelectronic techniques, the gate (43) of these electrodes being realised in n+ doped polysilicon and is common to all devices in a row (word line) the drain (41) of all devices in a column being connected together by using a metal contact plug and a metal line (44), the source (42) of the transistor being connected via a metal (usually tungsten) plug (46) to a gold layer (47) which acts as the active electrode.
15. Microelectrodes according to claim 11 consisting of a capacitive microelectrode obtained with an insulating substrate (60) a metal (61) and a thin insulating layer (64) said microelectrodes being separated by insulating material (62) and

covered in non exposed areas by a passivation layer (63).

16. Method of electroporation characterised in that an apparatus according to claims 1 – 3 is used.

17. Method according to claim 16 characterised in that performs one or more electroporations to at least a single adhering cell.

18. Method according to claim 17 characterised in that said apparatus comprises a biochip according to claims 4 - 11.

19. Method according to claims 16 - 18 characterised in that said biochip comprises microelectrodes according to claims 12 – 15.

20. Method according to claims 16 - 19 characterised in that the wave generator sends to the electrodes trains of pulses of various amplitude and duration.

21. Method according to claims 16 – 20 characterised in that the wave generator sends to the microelectrodes five trains of 25 pulses (1 ms duration) repeated at a time interval of 500 ms.

22. Method according to claims 16 – 20 characterised in that a trains of triangular voltages consisting of 10 pulses are applied to the electrodes the interval between one train and another being of 5 s.

23. Method according to any of preceding claims characterised in that it comprises substantially the following steps:

- cultivate cells since the adhering stage is reached
- add in the culture medium at least one compound to be electroporated in at least one single cell of the said cells
- selected at least one single cell and at least one microelectrode on which said selected single cell is adherent
- generate at least one electric signal suitable to electroporate said at least one single cell with said at least one compound to be electroporated and drive said electric signal to the said one microelectrode on which said selected single cell is adherent.

24. Electroporated cells characterised in that they are obtained with method according claims 16 - 23.

25. Electroporated cells according to claim 24 wherein the electroporated agents are drugs, genetic constructs and proteins.